mesothelial cells were visualized (data not shown). By the 4th week, metastases to the greater omentum, mesenterium, peritoneum, and lymph nodes were noted and some animals also showed additional metastasis to the diaphragm (Table 2). Metastasis to the liver was occasionally seen. In some mice, in which the tumors grew rapidly, formation of ascites began to be noted ~21 days after the orthotopic implantation. Some of these animals became moribund on the 28th day (Tables 1 and 2). By the 35th day, all the animals showed metastasis, with dissemination to the greater omentum, mesenterium, and peritoneum accompanied by the formation of bloody ascites as well as lymph node metastasis (Table 2). Metastasis to the diaphragm was also seen frequently. Micrometastasis to the kidneys was noted in a few animals.

Analysis of the progression of dissemination using luciferase gene–tranfected cells. The analytic method described above allows detailed evaluation even of micrometastases. However, it has limitations from the viewpoint of quantitative and objective analysis. To resolve these problems, we introduced the luciferase gene into the metastatic 44As3 cell line and its parent cell line HSC-44PE by means of liposome transfer; this yielded cells with high luciferase activity, 44As3Luc and HSC44Luc, respectively. When the 44As3Luc cells (1×10^6/100 μL) were implanted s.c. into nude mice, a significant correlation was observed between tumor growth (volume) and the luciferase emission level (photon number; Fig. 3). Both cell lines were therefore used for the subsequent experiments.

The metastatic 44As3Luc or its parent cell line HSC44Luc cells were implanted orthotopically into nude mice. With the light emission noted at the site of implantation, photon counting analysis was thereafter carried out at intervals of 3 or 4 days. Figure 4*A* (*top*) presents a typical example. Chronological observation of the same animals, which were kept alive, was possible by this method. The 44As3Luc cells proliferated actively in the stomach. By the 15th day after implantation, tumor invasion of the peritoneal cavity and gradual progression of dissemination and increases in the sizes of the cell clusters were observed. Around the 25th day after implantation, a marked increase in the volume of the ascitic pool was noted by macroscopic observation, and some moribund mice were observed after the 29th day. When the moribund animals were sacrificed for autopsy, dissemination to the mesenterium and parietal peritoneum was often observed, frequently accompanied by metastasis to the lymph nodes. It was confirmed anatomically and histopathologically that the light-emitting sites corresponded to the tumor-affected sites (Fig. 4*B*). On the other hand, in the animals transplanted with the HSC44Luc, the tumor growth tended to be confined to the region of the stomach where the cells had been implanted (Fig. 4*B*), with slower tumor cell proliferation. As shown in Fig. 4*A* (*bottom*), luminescence was sometimes noted in the lymph nodes around the stomach and so on, but all of these foci of metastasis had regressed by ~60 days after implantation. Moribund animals began to be observed by the 85th day, although no ascites formation was noted in any of the animals.

By plotting the number of photons against time, a tumor growth curve reflecting the progression of peritoneal dissemination was obtained. When the relative number of photons from the highly metastatic cell line 44As3Luc and its parent cell line HSC44Luc (relative to the number of photons immediately after transplantation = 100) was plotted against time, quantitative comparison of the extents of proliferation of the two cell lines with different metastasizing potentials was possible (Fig. 4*C*).

Evaluation of the possibility of quantitative and objective screening of the effectiveness of anticancer agents. In a previous study, tumor growth was found to be suppressed in animals given i.v. injections of CPT-11, resulting in a significant prolongation

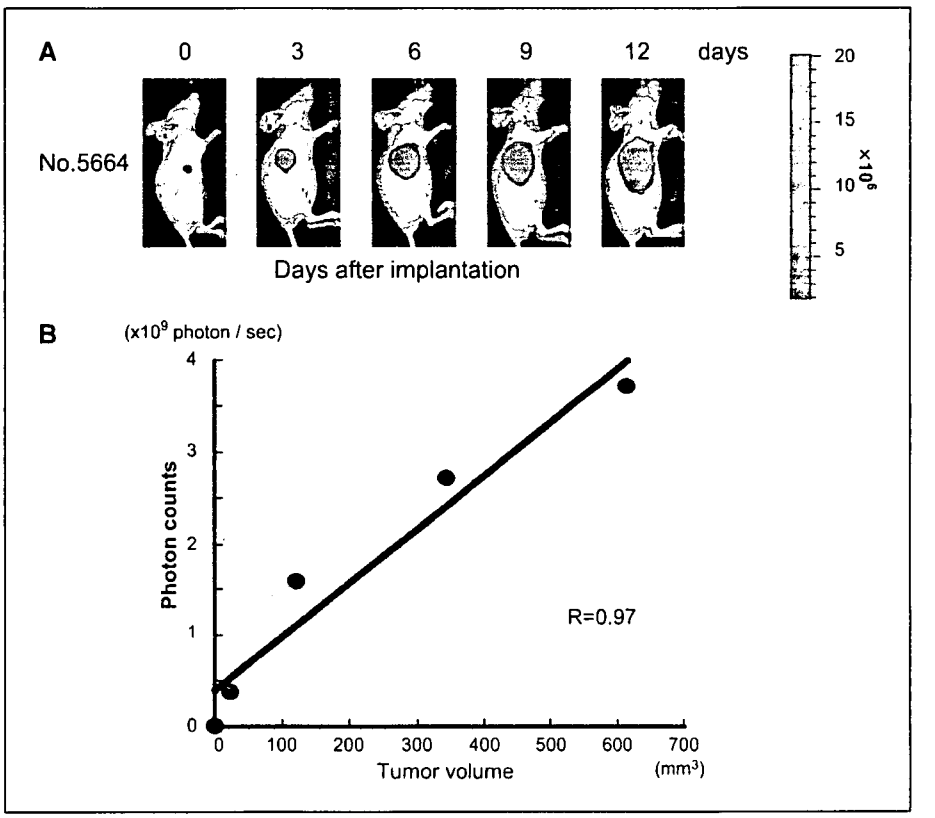

Figure 3. Correlation between the photon counts and tumor volume. *A*, nude mice bearing 44As3Luc tumors in the s.c. were visualized in anesthetized animals after i.p. inoculation of luciferin. *B*, correlation plot; strong correlation ($R = 0.97$) was observed between the conventional methods and our photon counting analysis method for monitoring the growth of a s.c. 44As3Luc tumor ($n = 5$). The tumor mass was measured at predetermined time intervals in two dimensions with calipers, and the tumor volume was calculated according to the equation $(l \times w^2) / 2$, where l is the length and w is the width (16).

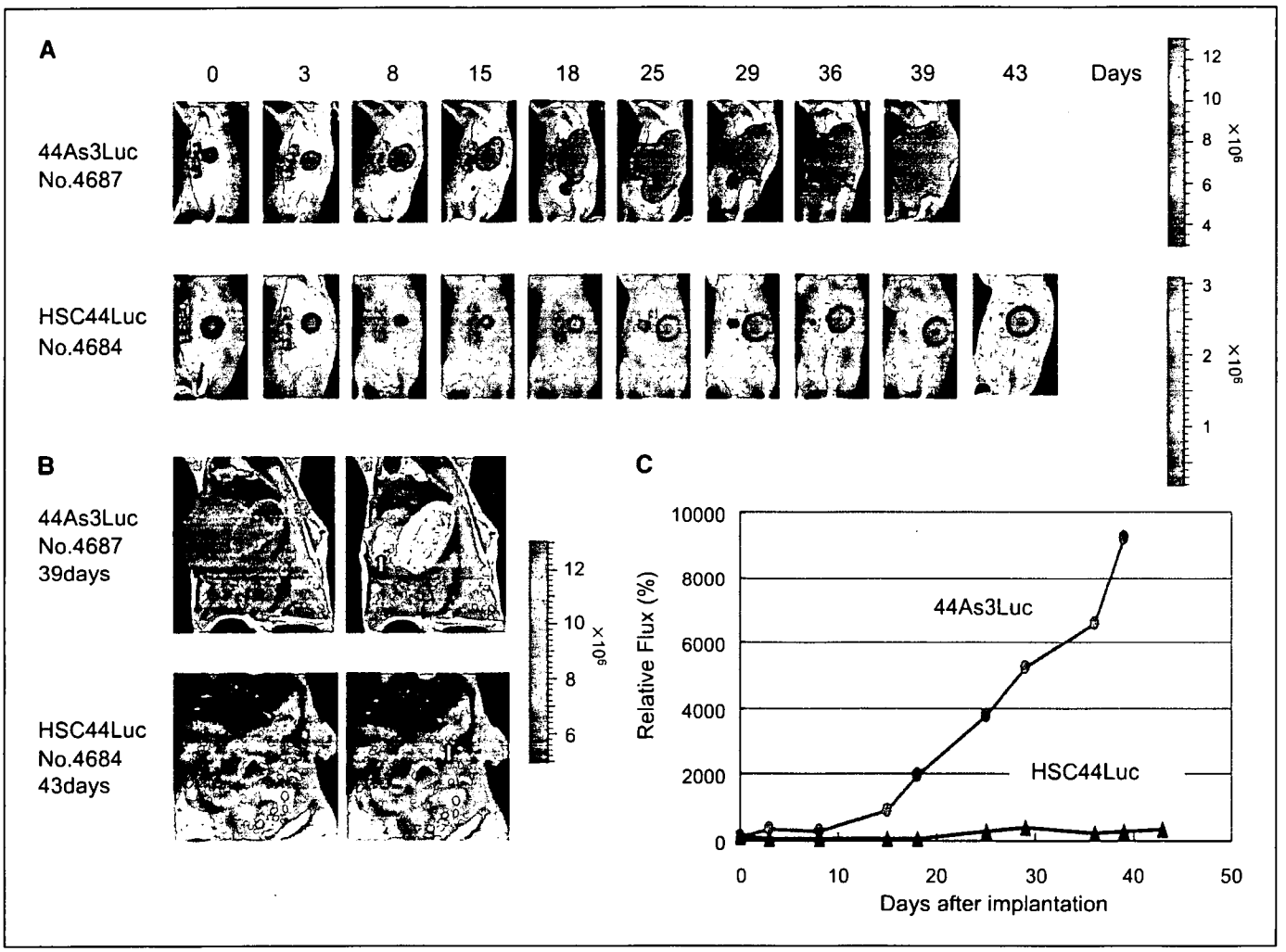

Figure 4. Quantitative photon counting analysis of progression process of peritoneal disseminated metastasis of the highly metastatic and the parent cell lines. *A*, detection of progression process of peritoneal disseminated metastasis. *B*, photon counting analysis of the peritoneal disseminations after orthotopic implantation (*yellow arrow*, site) of the cells. *C*, quantitative analysis of progression process of peritoneal disseminated metastasis of 44As3Luc (●) and HSC44Luc (▲) cell lines (n = 5). This experiment was repeated thrice, and similar results were observed each time.

of the survival period (17). A similar evaluation was conducted in the present study using 44As3Luc cells. Figure 5 (*top*) shows a typical example of the photon counting analysis, whereas Figure 5 (*bottom*) shows the time course of the changes in the number of photons. Following three doses of CPT-11 (200 mg/kg/mouse), the tumor gradually decreased in size, reaching a level close to the limit of detection on the 20th day. During the 5th and 6th week, the tumor began to show slow growth in the stomach followed during the 8th/9th week by peritoneal invasion and the onset of cancerous peritonitis accompanied by ascites formation and death of the animals. The survival period was markedly longer in the drug-treated group compared with that in the saline-treated controls. Plotting of the number of photons measured (average of five animals) against time yielded a tumor growth curve, thus allowing quantitative evaluation of drug-induced suppression of the progression of peritoneal dissemination (Fig. 5, *bottom*).

As stated above, the 44As3Luc cells began to proliferate again during the 5th/6th week after implantation in the CPT-11 treatment group. We therefore gave three additional doses beginning on day 28 (after the onset of reproliferation). Figure 5 (*top*) shows a typical example of the bioluminescence signal in such a case. The additional doses of CPT-11 (400 mg/kg/mouse) markedly suppressed the proliferation of the 44As3Luc cells until around day 60; however, proliferation again began to be detected thereafter. By around day 80, the tumor started to grow more rapidly and spread, causing moribund animals to appear by around day 90. The survival period of the animals was markedly prolonged by the additional drug doses. Figure 5 (*bottom*) shows the time course of changes in the number of photons (average of five animals). Quantitative comparison of the proliferation and spread of the tumor cells was possible between the drug treatment group and the control group and between two drug treatment groups, thus allowing objective evaluation of the responses to treatment.

Discussion

Before the present study, very little was known about how scirrhous gastric carcinoma cells invaded and proliferated within the primary lesion, how they exfoliated and thus became free, how they colonized and proliferated within the peritoneal cavity, or how they advanced to the stage of cancerous peritonitis. Herein, we investigated the course of proliferation and spread of gastric cancer

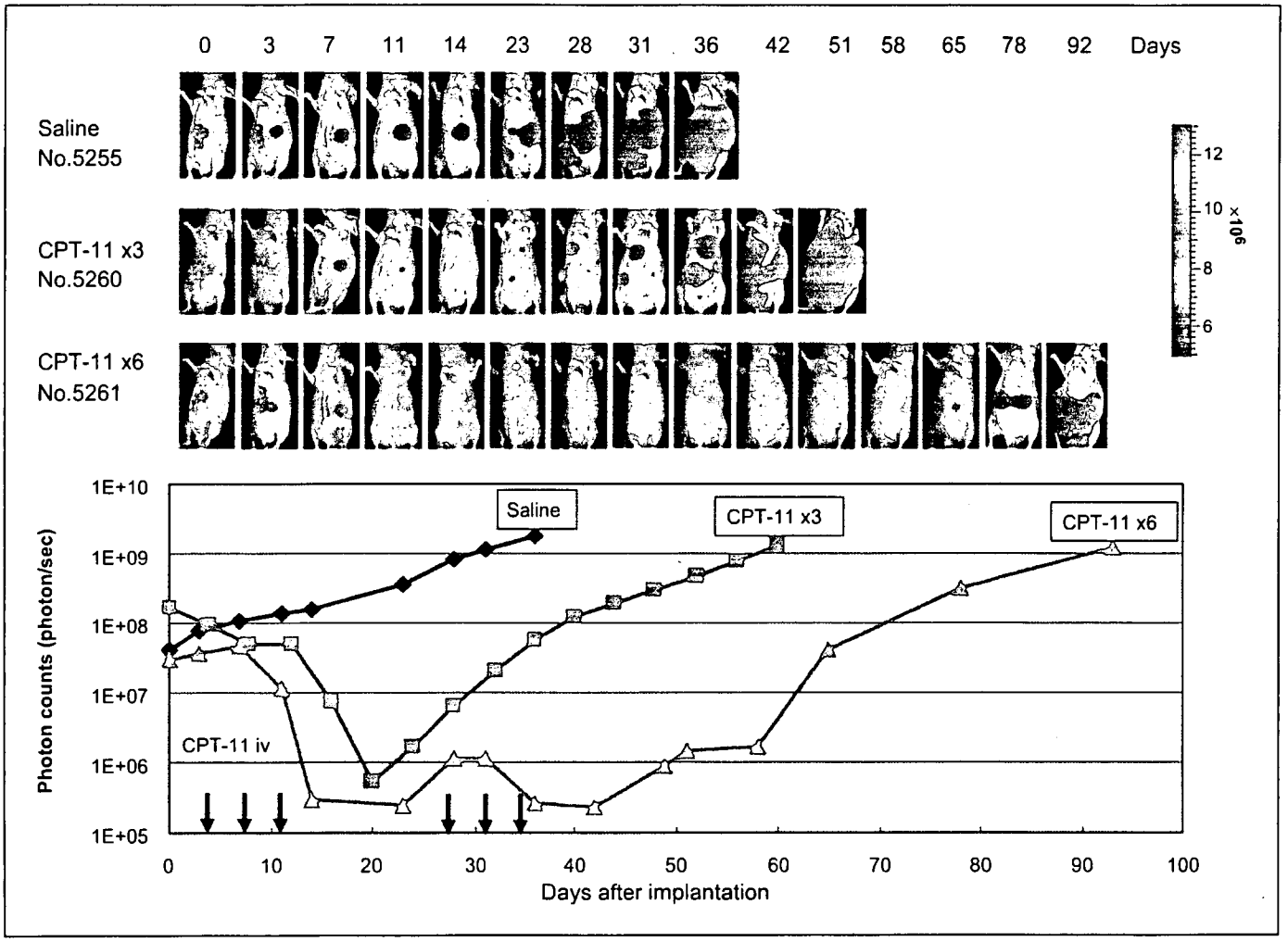

Figure 5. Quantitative photon counting analysis of the effect of CPT-11 on peritoneal disseminated metastasis 44As3Luc mouse model. Effects of CPT-11 in the peritoneal dissemination mouse model established using orthotopically implanted 44As3Luc cells. Mice receiving CPT-11 (*arrow*) or vehicle alone as control ($n = 5$; $P < 0.001$) were monitored twice weekly for the development of peritoneal dissemination. Similar results were obtained in a second experiment conducted independently.

cells by sacrificing the animals at different points of time after orthotopic implantation of the highly metastatic tumor cell line 44As3 (17) and conducted anatomic and histopathologic examinations in the sacrificed animals. In this experiment, the sequence of findings seems to endorse the previous contention that gastric cancer cells invade deeper layers of the gastric wall to reach the serosa and then exfoliate, thereby being released into the peritoneal cavity, resulting in peritoneal dissemination.

The growth of tumors in the gastric wall and the subsequent progression to cancerous peritonitis are difficult to monitor extracorporeally unlike s.c. tumors. For monitoring the progression of tumor dissemination, the only possible method was to implant the tumor cells into groups of mice and sacrifice the animals at different points of time for autopsy and observation; quantitative comparison was still not possible by this method (10–12, 18–25). All of these problems were resolved in the present study by introduction of the luciferase gene into tumor cells with a high metastasizing potential and subsequent *in vivo* photon counting analysis. In the first step, we confirmed that the results of the conventional method of evaluation in relation to proliferation of our gastric carcinoma cells were consistent with the results of our photon counting analysis. We then conducted an experiment on a model of peritoneal dissemination. Using the *in vivo* photon counting technique, it was possible to observe the same animals successively, beginning from the growth of the tumor at the site of implantation to peritoneal dissemination and, finally, the formation of ascites. Furthermore, it was possible to observe the processes of dissemination progression on a real-time basis, allowing quantitative analysis and comparison of the course of proliferation and progression within the living body after implantation of a cell line with high metastasizing potential and its parent cell line based on changes in the photon number.

Needless to say, it is important to develop a screening model for exploring substances effective against tumors and ultimately developing clinically useful anticancer agents. We previously reported that an animal model of peritoneal dissemination established using the highly metastatic cell lines (44As3, 58As1, and 58As9) established by our group satisfied all of the requirements of a model for drug screening (17, 44). However, before this model can be applied as a universally valid drug evaluation system, the following problems must be resolved: (*a*) methods for appropriate observation and objective evaluation are urgently needed,

(*b*) excellent operative skill is indispensable for orthotopic implantation with high reproducibility, and (*c*) large numbers of animals are needed. With the establishment of this experimental system, the conventional problems associated with the evaluation of peritoneal dissemination have been overcome and highly reliable data are now obtainable. Therefore, a stage has been reached where this model of peritoneal dissemination can also be applied as a system for evaluation of the effects of drugs. Furthermore, because photon counting analysis allows noninvasive evaluation of the fate of cancer cells *in vivo* on a real-time basis, the pain experienced by experimental animals may be reduced, such that this technique would also be useful from the viewpoint of animal welfare (45).

We have used the bioluminescence signal from the luciferase reporter gene in our peritoneal metastasis model. Luciferase genes in our tumor cells can function stably over significant periods in tumors and in their metastases. To date, several other peritoneal metastasis models of human stomach cancer in animals have been reported (28, 31). For example, Hasegawa et al. (28) used green fluorescent protein (GFP) retroviral-infected human stomach cancer. In this nude mouse model, tumor cells were peritoneally injected and GFP transduction allowed visualization of the subsequent metastatic process. A major advantage of GFP labeling is that imaging requires no preparative procedures and hence allows for direct visualization in living tissue (26, 27, 29, 32, 34). In contrast, photon counting technique requires exogenous injection of luciferin substrate, which can stress the animals, and in addition, the intensity of the luciferase signal may sometimes be variable and unstable (46). Furthermore, Ray et al. (32) reported that red fluorescent protein imaging is ~ 1,000 times stronger than that of luciferase *in vivo*. Therefore, for monitoring the tumor metastasis process at the single-cell level, fluorescence imaging may be the more practical method. In fact, fluorescence-based orthotopic metastatic models have been used to study mechanisms and for drug discovery (14, 30, 33, 35).

In conclusion, our photon counting analysis involving a highly metastatic cell line, 44As3Luc, seems to be a useful model for studies, such as those designed to clarify the mechanism of peritoneal dissemination progression in intractable scirrhous gastric carcinoma, and for the development of new agents effective against such tumors.

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Detection of Epidermal Growth Factor Receptor Mutations in Serum as a Predictor of the Response to Gefitinib in Patients with Non–Small-Cell Lung Cancer

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Abstract Cases of non–small-cell lung cancer (NSCLC) carrying the somatic mutation of epidermal growth factor receptor (EGFR) have been shown to be hyperresponsive to the EGFR tyrosine kinase inhibitor gefitinib (IRESSA). If EGFR mutations can be observed in serum DNA, this could serve as a noninvasive source of information on the genotype of the original tumor cells that could influence treatment and the ability to predict patient response to gefitinib. Serum genomic DNA was obtained from Japanese patients with NSCLC before first-line gefitinib monotherapy. Scorpion Amplified Refractory Mutation System technology was used to detect EGFR mutations. Wild-type EGFR was detected in all of the 27 serum samples. EGFR mutations were detected in 13 of 27 (48.1%) patients and two major EGFR mutations were identified (E746_A750del and L858R). The EGFR mutations were seen significantly more frequently in patients with a partial response than in patients with stable disease or progressive disease ($P = 0.046$, Fisher's exact test). The median progression-free survival was significantly longer in patients with EGFR mutations than in patients without EGFR mutations (200 versus 46 days; $P = 0.005$, log-rank test). The median survival was 611 days in patients with EGFR mutations and 232 days in patients without EGFR mutations ($P > 0.05$). In pairs of tumor and serum samples obtained from 11 patients, the EGFR mutation status in the tumors was consistent with those in the serum of 8 of 11 (72.7%) of the paired samples. Thus, EGFR mutations were detectable using Scorpion Amplified Refractory Mutation System technology in serum DNA from patients with NSCLC. These results suggest that patients with EGFR mutations seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients without EGFR mutations.

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future (1). Targeting the epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of non–small-cell lung cancer (NSCLC) as EGFR has been found to be expressed, sometimes strongly, in NSCLC tumors (2). Mutations of EGFR tyrosine kinase have been reported in NSCLC patients with dramatic responses to gefitinib (IRESSA), an EGFR tyrosine kinase inhibitor (3, 4). Studies have reported that EGFR mutations are strong determinants of tumor response to EGFR tyrosine kinase inhibitors (5–7). Approximately 30 mutations in exons 18 to 21 of EGFR were detected in a lung tumor specimen (3–8). The two most common NSCLC-associated EGFR mutations are the 15-bp nucleotide in-frame deletion in exon 19 (E746_A750del) and the point mutation replacing leucine with arginine at codon 858 in exon 21 (L858R; refs. 5, 8). These two mutations account for ~90% of all EGFR mutations and could explain the dramatic responders to gefitinib. Most EGFR mutations have been identified retrospectively from operative resected tumor samples. However, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies; thus, it is necessary to establish a method to detect mutant EGFR, especially the two major mutations, from other more readily accessible patient samples.

Recently, PCR technology for the amplification of small amounts of DNA has made it possible to identify the same alterations, which are typically observed in DNA from resected or biopsied tumor cells, using serum samples from patients with various types of tumor, including NSCLC (9, 10). The detection of EGFR mutations in serum DNA may provide a noninvasive and repeatable source of genotypic information

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that could influence treatment and prognosis, especially in patients with NSCLC treated with gefitinib. However, it is well known that interfusion of normal cells with tumor cells prevents the detection of mutations in the tumor cells. Therefore, it is necessary to enhance the sensitivity of the detection of EGFR mutations from tumor-derived DNA mixed with normal cells.

Scorpion primers are used in a fluorescence-based method for the specific detection of PCR products (11). A Scorpion is a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequences at the 5′ and 3′ ends of the probe. Scorpion can be used in combination with the Amplified Refractory Mutation System (ARMS) to enable the detection of single-base mutations (11, 12). ARMS technology is used for allele discrimination and additional mismatches are introduced near the 3′ terminus of the primers to enhance specificity. For the detection of known mutations, the Scorpion-ARMS method is highly sensitive and fast (13). Our hypothesis was that the ARMS and Scorpion methods could enhance the sensitivity of the detection of EGFR mutations from the wild type.

The aims of this study were to develop a highly sensitive assay for the detection of EGFR mutations in serum DNA, to compare the mutation status in serum to tumors from a subset of their patients, and to clarify the relationship between the EGFR mutation status in serum DNA and clinical manifestations, and in particular the responsiveness to gefitinib.

Materials and Methods

Patients and clinical trials. This study was carried out as a correlative study in a multicenter clinical phase II trial of gefitinib monotherapy at the Department of Respiratory Medicine, Kanzawa University Hospital; the Department of Internal Medicine, Kouseiren Takaoka Hospital; the Department of Internal Medicine, Shinminato Municipal Hospital; the Department of Internal Medicine, Fukuiken Saiseikai Hospital; the Department of Respiratory Medicine, Toyama City Hospital; the Department of Respiratory Medicine, Ishikawa Prefectoral Hospital; and the Department of Respiratory Medicine, Kanazawa Municipal Hospital. According to Simon's minimax design, our study, with a sample size of 25, had an 80% power to support the hypothesis that the true objective response rate was >30% and a 5% significance to deny the hypothesis that the true objective response rate was <10%. Assuming an inevaluability rate of <20%, we projected an accrual of 30 patients. The study was conducted with the approval of the appropriate ethical review boards based on the recommendations of the Declaration of Helsinki for biomedical research involving human subjects. Japanese patients with stage IIIB or IV histologically or cytologically proven chemotherapy-naïve NSCLC were enrolled in this trial. Gefitinib was orally given to all patients at a fixed dosage of 250 mg/d. Efficacy was assessed using the Response Evaluation Criteria in Solid Tumors guidelines (14). The analysis of the samples in this study was done blinded to the clinical outcome.

Blood sample collection and DNA extraction. Blood samples from the 27 patients with NSCLC were collected before the initiation of gefitinib administration. Separated serum was stocked at −80°C until use. Serum DNA was extracted and purified using a Qiamp Blood Kit (Qiagen, Hilden, Germany) with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The resulting DNA was eluted in 50 μL of sterile bidistilled buffer. The concentration and purity of the extracted DNA were determined by spectrophotometry. The extracted DNA was stocked at −20°C until use.

Scorpion ARMS primers for the detection of E746_A750del and L858R. We used an EGFR Scorpion Kit (DxS Ltd., Manchester, United Kingdom), which combined two technologies (i.e., ARMS and Scorpion) to detect mutations in real-time PCR reactions. Four kinds of scorpion primers for the detection of E746_A750del, L858R, and wild type in both exon 19 and exon 21 were designed and synthesized by DxS. The sequences of the scorpion primer for E746_A750del and L858R were based on the GenBank-archived human sequence for EGFR (accession no. AY588246). All reactions were done in 25-μL volumes using 1 μL of template DNA, 7.5 μL of reaction buffer mix, 0.6 μL of primer mix, and 0.1 μL of Taq polymerase. All reagents are included in this kit. Real-time PCR was carried out using SmartCycler II (Cepheid, Sunnyvale, CA) under the following conditions: initial denaturation at 95°C for 10 minutes, 50 cycles of 95°C for 30 seconds, and 62°C for 60 seconds with fluorescence reading (set to FAM that allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was done with Cepheid SmartCycler software (Ver. 1.2b). The cycle threshold (Ct) was defined as the cycle at the highest peak of the second derivative curve, which represented the point of maximum curvature of the growth curve. Both Ct and maximum fluorescence (Fl) were used for interpretation of the results. Positive results were defined as follows: Ct ≤45 and Fl ≥50. These analyses were done in duplicate for each sample and reviewed by two investigators blinded to any clinical information. To confirm the sensitivities for the detection of E746_A750del and L858R, we used the standard DNA that was included in the EGFR Scorpion Kit. Standard DNA with E746_A750del and L858R at a volume of 1, 10, 100, 1,000, or 10,000 pg and the mixture of standard DNA with wild type at 10,000 pg and standard DNA with E746_A750del and L858R at a volume of 1, 10, 100, 1,000 or 10,000 pg were used. For quantification, a standard curve was generated by plotting the cycle number of Ct against the log of the DNA volume of the known standards. The linear correlation coefficient (R^2) values and the formula of the slopes were calculated. DNA (10,000 pg) for the positive control was extracted from a Japanese human adenocarcinoma PC-9 cell line known to contain E746_A750del, a Japanese human adenocarcinoma 11_18 cell line known to contain L858R, and a human epidermoid carcinoma A431 cell line known to contain wild-type exon 19.

Tissue sample collection and DNA extraction. Tumor specimens were obtained on protocols approved by the Institutional Review Board. Twenty paraffin blocks of tumor material, obtained from 15 patients at the time of diagnoses (and before treatment), were collected retrospectively. Eleven tumor samples were collected from the primary cancer via transbronchial lung biopsy, one was resected intraoperatively, and nine were from metastatic sites (four from bone, three lymph nodes, one brain, and one colon). All specimens underwent histologic examination to confirm the diagnosis of NSCLC. DNA extraction from tumor samples was done using a DEXPAT kit (TaKaRa Biomedicals, Shiga, Japan).

PCR amplification and direct sequencing. Amplification and direct sequencing were done in duplicate for each sample obtained from serum and tissue specimens. PCR was done in 25-μL volumes using 15 μL of template DNA, 0.75 units of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 2.5 μL of PCR buffer, 0.8 mmol/L deoxynucleotide triphosphate, 0.5 μmol/L of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The sequences of primer sets and schedules of amplifications were followed as previously described (12). The amplification was done using a thermal cycler (Perkin-Elmer, Foster City, CA). Sequencing was done using an ABI prism 310 (Applied Biosystems, Foster City, CA). The sequences were compared with the GenBank-archived human sequence for EGFR (accession no. AY588246).

Statistical analysis. Fisher's exact test was used to assess the relationship between the presence of EGFR mutations in patients with NSCLC and different characteristics, including gender, tumor histology, and response to gefitinib. Regarding analyses of response to gefitinib, patients were categorized into the two groups: (*a*) partial response and (*b*) stable disease or progressive disease (Response Evaluation Criteria

in Solid Tumors criteria). We compared Kaplan-Meier curves for overall survival and progression-free survival using the standard log-rank test. Overall survival was defined as the time from the initiation of gefitinib administration to death from any cause; patients known to be still alive at the time of the analysis were censored at the time of their last follow-up. Progression-free survival was defined as the time from the initiation of gefitinib administration to first appearance of progressive disease or death from any cause; patients known to be alive and without progressive disease at the time of analysis were censored at the time of their last follow-up. $P = 0.05$ was considered statistically significant. The statistical analyses were done using the StatView software package version 5.0.

Results

Patients and extracted DNA from serum. Twenty-eight patients were enrolled between October 23, 2002 and August 3, 2003 (Table 1). All patients were evaluated for response and followed for progression-free survival and overall survival. Blood samples (2 mL) were collected from 27 of these patients before the initiation of gefitinib administration. These 27 patients represented a subset of that phase II study. Serum DNA was extracted in all 27 samples at a median concentration of 70.0 ng/mL (range, 0-1,720.0 ng/mL).

Sensitivity of the EGFR Scorpion. Preliminary experiments were done to evaluate the sensitivity of the EGFR Scorpion kit (Fig. 1A-C). All curves using E746_A750del and L858R standard DNA (volumes of 1-10,000 pg) increased up to 45 cycles (Fig. 1A). When wild-type standard DNA and distilled water were used as negative controls, the curves did not increase and continued flat at 50 cycles (Fig. 1A and C). When diluted E746_A750del and L858R standard DNA were mixed with wild-type standard DNA at ratios from 10^0 to 10^{-5}, all curves that indicated the presence of E746_A750del and L858R increased up to 45 cycles (Fig. 1B and D). Standard curves in the range of measured volumes in this study were linear with r^2 values from 0.987 to 0.998. Both slopes of curves were almost parallel (Fig. 1E). The Ct of diluted mutant standard DNA mixed with wild-type DNA was close to that of mutant standard DNA alone. Although the peak fluorescence levels of diluted E746_A750del standard DNA mixed with wild-type DNA were lower than without wild-DNA standard, the presence of E746_A750del was clearly detected at ratios less than 10^{-4}. The peak fluorescence levels of diluted L858R standard DNA mixed with wild-type DNA were equivalent to those without wild-DNA standard. Curves of DNA with the mutations at an amount of up to 1 pg were unaffected by interfusion of DNA of wild-type EGFR. There were no significant differences between either the minimum detectable volume of the mutations or the minimum detectable ratio of wild type to the mutations.

In the cell-based experiments using genomic DNA of human cancer cell lines, the signal using DNA derived from the PC-9 cells was detected whereas the signal using DNA from the A431 cells was, as expected, not detected (Fig. 1D and E).

EGFR mutation status of serum DNA detected by EGFR scorpion. The E746_A750del or L858R status of serum DNA derived from 27 patients with NSCLC was examined. Wild-type exons 19 and 21 were detected from all serum samples. E746_A750del was detected in samples of 12 patients. L858R was detected in 1 patient (Table 2). In total, EGFR mutations were detected in 13 of 27 (48.1%) patients. The histologic subtypes of the original tumors are summarized in Table 3A in the 27 patients who were assessed for EGFR mutation in serum. Eleven of 23 (47.8%) cases of adenocarcinoma, one of two cases of squamous-cell carcinoma, and one of two cases of large-cell carcinoma were positive for EGFR mutations. An EGFR mutation was more frequently detected in the samples from female patients than those from males [7 of 10 (70%) versus 6 of 17 (35%); Table 3B].

EGFR mutation status in serum (EGFR Scorpion) and response to gefitinib. EGFR mutations were more frequently observed in the samples from the patients who showed a partial response (7 of 9 cases, 77.8%) than in samples from patients with stable disease or progressive disease (6 of 18 cases, 33.3%; $P = 0.046$, Fisher's exact test; Table 3C).

EGFR mutation status in serum (EGFR Scorpion) and effect on survival. Median progression-free survival and overall survival of all the patients treated with gefitinib were 98 and 306 days, respectively. Patients with EGFR mutations in serum showed a significantly longer median progression-free survival compared with the patients without EGFR mutations (200 versus 46 days, $P = 0.005$; Fig. 2A). The patients with EGFR mutations showed a longer median overall survival compared with the patients without EGFR mutations, although there was no statistical significance (611 versus 232 days, $P = 0.078$; Fig. 2B). These results suggest that patients who were serum EGFR mutation positive seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients who were EGFR mutation negative.

EGFR mutation in serum analyzed by direct sequencing and in comparison with EGFR Scorpion. The deletional mutation (E746_A750del) was detected by direct sequencing in serum DNA extracted from 10 of 27 patients (37.0%). No point mutation in exons 18, 19, and 21 was detected in the PCR

Table 1. Patient characteristics

	(*n*)
No. patients	27
Age (y)	
Median	64
Range	44-87
Sex	
Male	17
Female	10
Performance status	
0	19
1	6
2	2
Stage	
IIIB	3
IV	24
Histology	
Adenocarcinoma	23
Squamous-cell carcinoma	2
Large-cell carcinoma	2
Response	
Partial response	9
Stable disease	8
Progressive disease	10

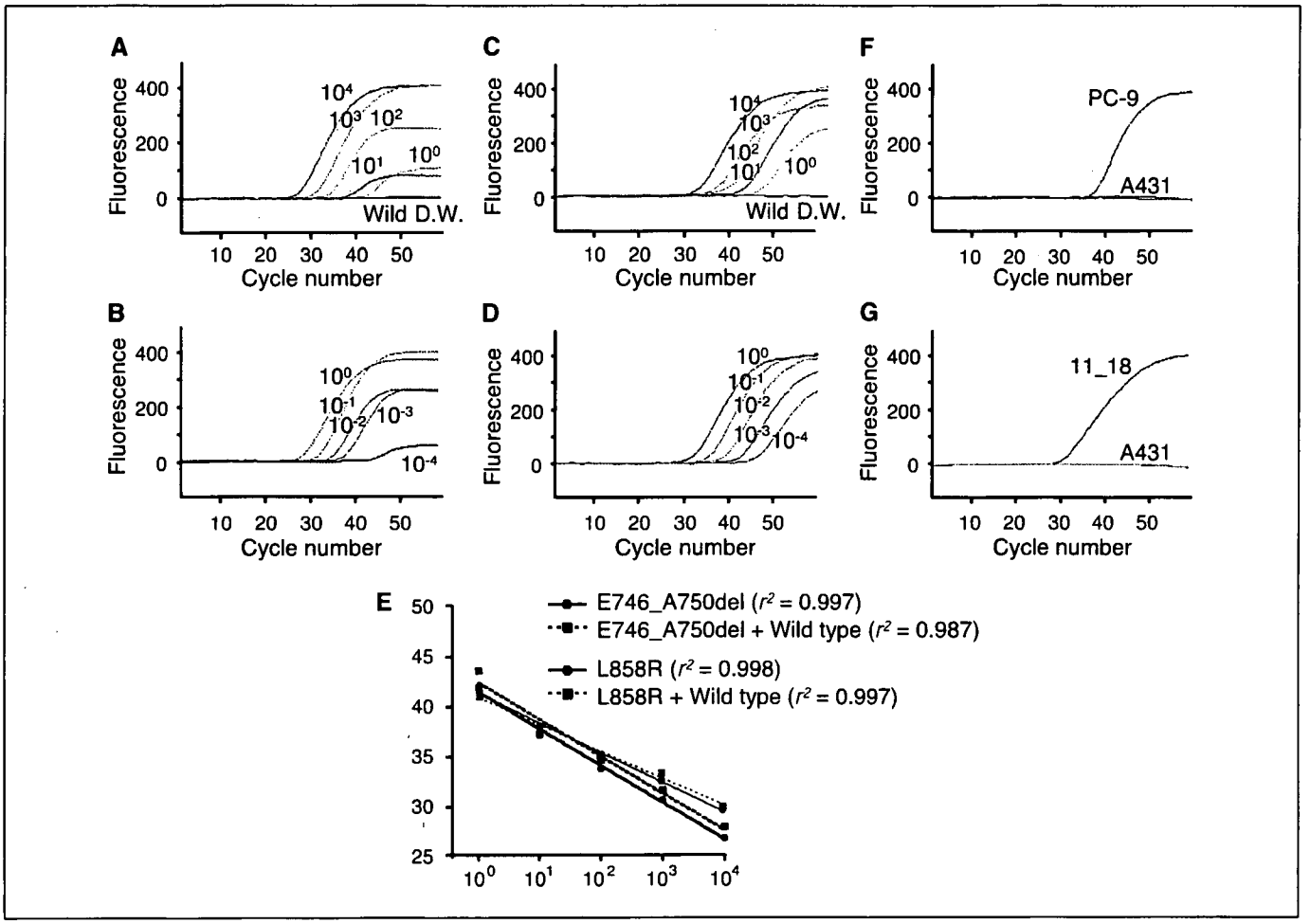

Fig. 1. Sensitivity of detection for mutations of E746_A750del and L858R using the EGFR Scorpion kit (*A* and *B*, E746_A750del; *C* and *D*, L858R). Standard DNA with E746_A750del (*A*) and L858R (*C*) were used at various volumes of 10,000 pg (10^4), 1,000 pg (10^3), 100 pg (10^2), 10 pg (10^1), and 1 pg (10^0). Standard DNA with wild-type (*Wild*) and distilled water (*D.W.*) were used as negative controls in the same experiment. Standard DNA with E746_A750del (*B*) and L858R (*D*) at concentrations from 1 to 10,000 pg were mixed with 10,000 pg of standard DNA with wild-type at a ratio of 1:1 (10^0), 1:10 (10^{-1}), 1:100 (10^{-2}), 1:1,000 (10^{-3}), and 1:10,000 (10^{-4}). *E*, standard curves were derived by plotting the Ct of each curve (shown in *A-D*) against the log of the standard DNA volume (*black lines*, E746_A750del; *blue lines*, L858R). *F*, PC-9 with E746_A750del and A431 with wild-type. *G*, 11_18 with L858R and A431.

products from serum samples. The serum EGFR status detected by direct sequence was not correlated statistically with histologic type, gender, response to gefitinib (Table 3), or survival (progression-free survival, $P = 0.277$; overall survival, $P = 0.859$). EGFR mutation status, as assessed by direct sequence, was consistent with those assessed by EGFR Scorpion in 15 of 27 (55.6%) of the paired samples. In four cases, EGFR mutation status (E746_A750del) was positive by direct sequence and negative by EGFR Scorpion. Eight cases were negative by direct sequence and positive by EGFR Scorpion. Thus, the sensitivity of EGFR Scorpion seems to be higher than that of direct sequencing due to the use of the specific primers for EGFR mutations in this kit.

EGFR mutations in tumors in comparison with those in serum. Twenty tumor samples were obtained from 15 patients retrospectively. Sequencing of EGFR exons 19 and 21 was done in samples from 12 of these under the same PCR conditions (Table 4; the other three samples were not evaluated because of low amplification of PCR products). EGFR mutations were detected in four cases (25.0%); three were the 15-bp deletion (E746_A750del) in exon 19 and one was the L858R point mutation in exon 21. Tumor histology of patients with EGFR mutations was adenocarcinoma in three and large-cell carcinoma in one. The responses to gefitinib in these four patients were two partial response, one stable disease, and one progressive disease.

Pairs of tumor samples and serum samples were obtained retrospectively from 11 patients (Table 4). The EGFR mutation status in the tumors was consistent with those in the serum of 8 of 11 (72.7%) of the paired samples. The E746_A750del mutation was positive in the tumor and negative in the serum in two patients, and the E746_A750del mutation was negative in the tumor and positive in the serum in one patient.

Discussion

Our findings have shown that EGFR mutations were detectable in serum samples obtained from patients with NSCLC and that the EGFR Scorpion kit consisting of ARMS and Scorpion technology is a useful method for detection of EGFR mutations. The EGFR mutation status in serum detected by the EGFR Scorpion was correlated statistically with responsiveness to, and the progression-free survival of, gefitinib treatment. Our finding supports the hypothesis that the EGFR

mutation status from serum DNA is useful to predict the responsiveness to gefitinib.

The mutation rate observed in our study seems to be relatively high (48%) although we have detected only two major mutations. EGFR mutations have been detected at a higher frequency in lung tumors from female patients, those with adenocarcinoma histology, nonsmokers, and patients of Asian origin (6, 8). However, previous reports show that the mutation rate of EGFR in operative samples of Japanese patients was from 26% to 59% (4, 6, 15, 16). The EGFR mutation rate in our study is equivalent to that observed in these reports. It can be speculated that the high sensitivity and specificity of the EGFR Scorpion allowed us to detect the EGFR mutations even in serum. Another possible reason is the high number of patients with adenocarcinoma in our study (23 of 27, 85.2%). Previous studies have shown that very few patients with nonadenocarcinoma, including squamous cell carcinomas and large-cell carcinomas, have EGFR mutations (3–8). Our results were in line with the previous studies and showed that no patients with squamous cell carcinoma or large-cell carcinoma had the mutations.

Table 2. Patients' characteristics and EGFR mutant status detected from serum DNA using the EGFR ARMS-Scorpion method

Response	Gender	Histology	Exon 19		Exon 21	
			Wild	E746_A750del	Wild	L858R
PR	M	Ad	+	–	+	+
PR	F	Ad	+	+	+	–
PR	M	Ad	+	–	+	–
PR	F	Ad	+	+	+	–
PR	M	Ad	+	+	+	–
PR	F	Ad	+	–	+	–
PR	M	Ad	+	+	+	–
PR	F	Ad	+	+	+	–
PR	F	Ad	+	+	+	–
SD	M	Large	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Ad	+	–	+	–
SD	F	Ad	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Ad	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Scc	+	+	+	–
PD	F	Scc	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Large	+	+	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	+	+	–
PD	M	Ad	+	–	+	–

Abbreviations: SD, stable disease; PD, progressive disease; PR, partial response; M, male; F, female; Ad, adenocarcinoma; Large, large-cell carcinoma; Scc, squamous-cell carcinoma; +, curve detected by SmartCycler; –, curve not detected by SmartCycler.

Table 3. Frequency of EGFR mutations in serum DNA from patients with NSCLC according to histology (A), gender (B), and response to gefitinib (C)

	EGFR Scorpion kit			Direct sequence		
	+	–		+	–	
(A) Histology and EGFR mutant states						
Ad	11	12		8	15	
Non-Ad	2	2	$P > 0.999$	2	2	$P > 0.999$
(B) Gender and EGFR mutant states						
Female	7	3		5	5	
Male	6	11	$P = 0.120$	5	12	$P = 0.415$
(C) Response to gefitinib and EGFR mutant states						
PR	7	2		4	5	
SD/PD	6	12	$P = 0.046$	6	12	$P = 0.683$

NOTE: A total of 27 samples were obtained from 28 patients before treatment.

We identified 12 deletion mutations and a single point mutation (L858R). Previous reports have shown that the frequency of detection of E746_A750del is almost equivalent to that of L858R (15, 16). It seems that the rate of detection of L858R in our study was very low compared with the rate of E746_A750del. The sensitivity for detection of L858R using the Scorpion ARMS method is very high and equivalent to that of E746_A750del. We thus consider that it is unlikely that the low-frequency L858R mutation could be due to assay-related false-negative findings. On the other hand, it also seems unlikely that either sampling method or the patients' eligibility criteria are biased toward the high rate of E746_A750del. Therefore, we have not been able to clarify the moot point. Further analyses in much larger groups of patients will be necessary to clarify the frequency of the major two mutations in serum DNA. Unfortunately, parallel tumor tissue investigations were done only on a small subset of the participating patients. Furthermore, findings in the serum were divergent from those obtained from the primary tissue in 3 of 11 patients from whom the paired samples were obtained. Therefore, this study is at best hypothesis-forming and will require follow-up analysis in much larger groups of patients.

Some investigators reported that mutations in the EGFR tyrosine kinase domain enhanced responsiveness to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib, and seemed to be associated with the prolonged survival of the patients who received these drugs (7, 8). In a placebo controlled study showing a survival advantage for NSCLC patients who received erlotinib, Tsao et al. (17) showed that the presence of an EGFR mutation might increase responsiveness to erlotinib, but was not indicative of a survival benefit, and concluded that EGFR mutation analysis was not necessary to identify patients in whom treatment with EGFR inhibitors was appropriate. Our results are not in line with their conclusions. In their study, the rate of mutation analysis was low and 107 of 731 patients

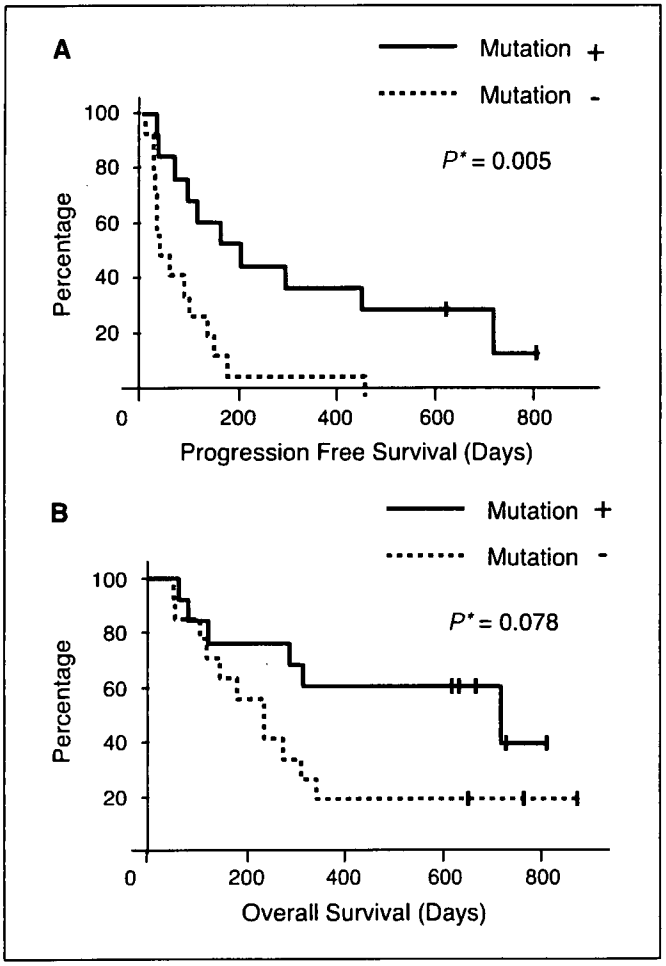

Fig. 2. Progression-free survival (*A*) and overall survival (*B*) with respect to the EGFR mutation status of NSCLC. *, log-rank test.

enrolled in their study were successfully analyzed for EGFR mutation. Sensitivity for detecting EGFR mutation in their study might be unstable as interfusion of normal cells in tumor cells decreases the sensitivity for detecting tumor-derived mutations using direct sequencing. They propose that additional processes (such as microdissection) to enrich tumor cell DNA might increase the rate of detection of new mutations; however, it seems that their results are insufficiently robust to reach this conclusion. Therefore, we propose the use of EGFR mutation analysis from serum DNA, which is easily collected and repeatable, to show that EGFR mutation status using the EGFR Scorpion kit correlates with the responsiveness to gefitinib.

EGFR mutation in NSCLC is reported to be somatic (3, 4). It is well known that the concentration of free circulating DNA in serum is higher in patients with tumors than in healthy volunteers (18) and it seems that the detected mutational EGFR in serum was tumor derived. This is the first report analyzing EGFR mutations from serum DNA and evaluating EGFR mutation status and clinical outcome (response and survival) with gefitinib. No other studies have analyzed EGFR mutations from samples other than actual tumor samples. The mutation in two patients was positive in the tumor and negative in the serum, and the mutation in one patient was negative in the tumor and positive in the serum. We have tried to explain the discrepancy why tumor and serum were not better correlated as follows. In cases of positive in the tumor and negative in the serum, the volumes of mutant DNA extracted from the serum were under the detectable limit using the Scorpion ARMS method, or a very small amount of DNA derived from an actual tumor was circulating in the blood-stream. A previous study showed that 73% of patients with at least one molecular event, such as a hypermethylation of the tumor suppressor gene *p16*, in their tumor DNA had the same alteration in plasma DNA (10). In a case of negative in the tumor and positive in the serum, wild-type DNA interfered with

Table 4. EGFR mutation status in tumor samples and serum samples. Pairs of both tumor samples and serum samples were obtained from 11 patients

Gender	Histology	Response	EGFR mutation status				
			Tumor sample	EGFR Scorpion kit (serum sample)			
				Exon 19		Exon 21	
				Wild	Mutation	Wild	Mutation
M	Large	SD	Wild	+	–	+	–
F	Scc	PD	Wild	+	–	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	PR	L858R	+	–	+	+
F	Ad	SD	Wild*	+	+	+	–
M	Large	PD	E746-A750del	+	+	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	SD	E746-A750del*	+	–	+	–
F	Ad	PR	E746-A750del*	+	–	+	–
M	Ad	PD	Wild	+	–	+	–

*Patients who have different states of EGFR mutation from tumor-derived DNA and serum-derived DNA.

the detection of mutant DNA in the tumor samples using the direct sequencing method. The rate of the mutations in serum DNA detected by the Scorpion ARMS was compared with that in tumor tissues detected by the direct sequencing method as a current standard method. DNA from tumor samples consisted of a mixture of the mutant DNA and wild-type DNA because the EGFR mutation status was always heterogeneous, and the complete removal of normal cells, such as normal epithelial cells and inflammatory cells, from tumor specimens is very difficult. Parallel tumor tissue investigations were done on only a small subset of these patients, which is a recognized limitation in the present study. A larger study is necessary to evaluate the consistency of the mutation status from tumor and serum. On the other hand, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies. We showed that patients who were EGFR mutation positive in the serum DNA using the Scorpion ARMS method seem to have better outcomes with gefitinib treatment in terms of progression-free survival, overall survival, and response, despite the nonconformity between the mutation states of tumor and serum DNA in some of the patients. We anticipate that the detection of EGFR mutations in serum DNA using the Scorpion ARMS will be equivalently useful as a feasible approach for predicting tumor response to gefitinib.

Two groups have reported alternative methods for detection of EGFR mutations. One group used the LightCycler PCR assay (19) and the other postulated that the SSCP assay was more sensitive than direct sequencing and was a rapid method (20). Further studies are needed to validate these assays for detection of EGFR mutations and to clarify the most sensitive assay. Although the direct sequence method is common in reported studies, the EGFR mutation status in serum DNA by direct sequencing did not correlate with the responsiveness to and survival benefit of gefitinib in our study. These results indicate that the EGFR Scorpion kit is superior to the direct sequencing method for detection of an EGFR mutation in serum as a predictive marker.

One limitation of the EGFR Scorpion kit is that it is only able to detect mutations targeted by the designed Scorpion primers. EGFR mutations are not solely at these two sites but clustered around the ATP-binding site in exons 18, 19, and 21 (3–8). Moreover, the secondary mutation, a substitution of methionine for threonine at position 790 (T790M), leads to gefitinib resistance in NSCLC patients who have EGFR mutations and are responsive to treatment with gefitinib (21–23). Mutations in *K-ras*, a known downstream signaling molecule in the EGFR signaling pathway, are more frequent in patients who develop disease progression with treatment with either gefitinib or erlotinib (24). These mutation states may also be critical factors for the treatment of gefitinib. To clarify the usefulness of serum DNA as a source of genotypic information, the Scorpion primers need to be designed for detection of these mutations, and further studies using these primers are required.

In conclusion, the two major mutations of EGFR, E746_A750del and L858R, were detected in serum DNA with the EGFR Scorpion kit from patients with NSCLC. These results suggest that patients who were EGFR mutation positive seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients who were EGFR mutation negative. In the near future, a controlled clinical trial is necessary to confirm these conclusions.

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High sensitivity detection of *epidermal growth factor receptor* mutations in the pleural effusion of non-small cell lung cancer patients

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Epidermal growth factor receptor (EGFR) **mutations are a strong determinant of tumor response to gefitinib in non-small cell lung cancer (NSCLC). We attempted to elucidate the feasibility of *EGFR* mutation detection in cells of pleural effusion fluid. We obtained 24 samples of pleural effusion fluid from NSCLC patients. The pleural effusion fluid was centrifuged, and the cellular components obtained were used for detection. *EGFR* mutation status was determined by a direct sequencing method (exons 18–21) and by the Scorpion Amplified Refractory Mutation System (ARMS) method. *EGFR* mutations were detected in eight cases. Three mutations were detected by both methods, and the other five mutations were detected by Scorpion ARMS alone. The mutations were detected by both methods in all four partial responders among the seven patients who received gefitinib therapy. Direct sequencing detected the mutations in only two of four cases with partial response. These results suggest that the DNA in pleural effusion fluid can be used to detect EGFR mutations. The Scorpion *ARMS* method appears to be more sensitive for detecting *EGFR* mutations than the direct sequencing method. (*Cancer Sci* 2006; 97: 642–648)**

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future.[1] Targeting the epidermal growth factor receptor (EGFR) is one appealing strategy for the treatment of non-small cell lung cancer (NSCLC), because EGFR has been found to be expressed, sometimes strongly, in NSCLC.[2] Mutations of *EGFR* tyrosine kinase in NSCLC and hyper-responsiveness to gefitinib, a selective EGFR tyrosine kinase inhibitor, have been reported.[3,4] These mutations consist of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19 and 21 of *EGFR*, and increase the affinity of the enzyme for ATP and gefitinib. Some investigators have subsequently found that *EGFR* mutations are a strong determinant of tumor response to EGFR tyrosine kinase inhibitor.[5–7] Approximately 90% of the NSCLC-associated EGFR mutations in two reports consisted of two major *EGFR* mutations (E746_A750del in exon 19 and L858R in exon 21).[5,8] These investigators used surgical tissue to detect the *EGFR* mutations in their trials. As it is often difficult to obtain a tumor sample from patients with inoperable NSCLC, a method of detecting mutant *EGFR*, especially the two major mutations, in other specimens needs to be established.

Malignant pleural effusion is a common complication of lung cancer and is present in approximately 15% of lung cancer patients[9] and in 10–50% of patients at the time of diagnosis.[10] Approximately one-half of NSCLC patients with pleural effusion are initially positive cytologically, and most of the effusions are ultimately determined to be malignant. As sampling of pleural effusion fluid is usually easy, non-invasive and repeatable, we hypothesized that tumor cells in the pleural effusion fluid of NSCLC patients are a source of useful information on the status of the *EGFR* gene with regard to gefitinib response.

Genomic polymerase chain reaction (PCR) and the direct sequencing method have been used widely to detect *EGFR* mutations. It is well known that fusion between normal cells and tumor cells prevents detection of mutations in tumor cells by the direct sequencing method. Therefore it is necessary to enhance sensitivity for detection of *EGFR* mutations in a mixture of normal and tumor cells. We hypothesized that Scorpion Amplified Refractory Mutation System (ARMS) technology enhances sensitivity for detecting *EGFR* mutations. Scorpion primers are used in a fluorescence-based method for specific detection of PCR products.[11] A 'scorpion' consists of a specific probe sequence held in a hairpin loop configuration by complementary stem sequences on the 5′ and 3′ sides of the probe. A scorpion can be used in combination with ARMS to enable detection of single-base mutations.[11,12] The ARMS method was used for allele discrimination, and additional mismatches were introduced near the 3′ terminus of the primers to enhance specificity. A previous study showed that the ARMS method is superior to the direct sequencing method and the WAVE® (Transgenomic Inc., Cambridge, MA, USA) method for the detection of *EGFR* mutations.[13]

In the present study we attempted to detect major *EGFR* mutations in pleural effusion, and to find out whether the Scorpion ARMS method enhances sensitivity for detection of EGFR mutations in mixtures of DNA from normal cells and tumor cells.

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Patients and Methods

Patients

We studied NSCLC patients who had a pleural effusion at the time of diagnosis. The diagnosis of NSCLC was based on histological or cytological findings. This study was approved by the Institutional Review Boards of the National Cancer Center Hospital and Kanazawa University Hospital, and written informed consent was obtained from all participants. The patient record consisted of age, sex, smoking habit, histological type of NSCLC and treatment. The response of the patients treated with gefitinib was evaluated in accordance with the 'Response Evaluation Criteria in Solid Tumors (RECIST)' guidelines.[14] No research results were entered into the patient's record or released to the patient or their physician.

Collection of pleural effusion fluid and cell separation

The pleural effusion fluid was collected from patients in heparinized tubes between 29 March and 25 November 2005. No particular collection method was used. Pleural effusion fluid (1 mL) was centrifuged at 250 *g* for 10 min, and the cell pellet was stored at −80°C until use.

DNA extraction

DNA was extracted from the stored cell pellets using a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the protocol for tissue samples in the manufacturer's instructions. The DNA obtained was eluted in 50 μL of sterile bidistilled buffer, and the concentration and purity of the extracted DNA were assessed by spectrophotometry. The extracted DNA was stored at −20°C until use.

PCR amplification and direct sequencing

Exons 18, 19, 20 and 21 of the *EGFR* gene were amplified by PCR. The primers were designed based on a report by Lynch *et al.*[3] Genomic PCR of 20 ng of template DNA was carried out in 25-μL volumes containing 0.75 IU of Ampli *Taq* Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA), 2.5 μL of PCR buffer, 0.8 μM dNTP, 0.5 μM of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The first PCR analyses were carried out in a volume of 25 μL for 25 cycles, consisting of a denaturation step at 94°C for 45 s, a primer annealing step at 58°C for 30 s, and an elongation step at 72°C for 30 s. The final step at 72°C was extended for 10 min. Nested PCR was carried out for 20 cycles under the same conditions as the first PCR. Sequencing of each sample was carried out in duplicate using an ABI prism 310 (Applied Biosystems, Foster City, CA, USA). The sequences were compared with the GenBank-archived human sequence for EGFR (accession number AY588246).

Scorpion ARMS for the detection of E746_A750del and L858R

We used an EGFR Scorpion™ Kit (DxS, Manchester, UK), which combines the two technologies ARMS and Scorpion, to detect mutations in real-time PCR reactions. All reactions were carried out in 25-μL volumes with 1 μL of template DNA, 7.5 μL of reaction buffer mix, 0.6 μL of primer mix and 0.1 μL of *Taq* polymerase. Real-time PCR was carried out using SmartCycler® II (Cepheid, Sunnyvale, CA, USA) under the following conditions: initial denaturation at 95°C for 10 min, 50 cycles of 95°C for 30 s, and 62°C for 60 s with fluorescence reading (set to FAM that allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was carried out using Cepheid SmartCycler software (version 1.2b). The threshold cycle (Ct) was defined as the cycle at the highest peak of the second derivative curve, which represents the point of maximum curvature of the growth curve. Positive results were defined as Ct 45 and maximum fluorescence intensity 50. Analysis of each sample was carried out in duplicate. The EGFR Scorpion Kit is intended for detection of the two major somatic mutations in *EGFR*. These mutations consist of an in-frame deletion in exon 19 (E746_A750del) and a point mutation in exon 21 (L858R). There are two types of E746_A750del, with starting points at nucleotide positions 2235 and 2236 (NM_005228). The assay can detect both types of E746_A750del. Other deletion patterns in exon 19 and other mutations in the tyrosine kinase domain of EGFR, which are also associated with sensitivity of lung cancers to gefitinib, can not be detected using this assay.

Experiments comparing the detection of E746_A750del in mixtures of wild-type and E746_A750del DNA by direct sequencing and Scorpion ARMS

We used the standard DNA included in the EGFR Scorpion Kit to confirm sensitivity for the detection of E746_A750del. The following DNA mixtures were prepared: 10, 100, 1000 and 10 000 pg E746_A750del DNA, and 10 000 pg wild-type DNA with 10, 100, 1000 and 10 000 pg E746_A750del DNA. These DNA mixtures were used to evaluate the sensitivity of direct sequencing and Scorpion ARMS. The results obtained using Scorpion ARMS were quantified using a standard curve generated by plotting the Ct against the log of the amount of DNA contained in the known standards. The linear correlation coefficient (R^2) values and the formulas for the slopes were calculated. To validate this assay we carried out the assay using plasmid DNA derived from the PCR products of A431 cells, which are known to contain wild-type DNA, PC-9 cells, which are known to contain E746_A750del, and 11-18 cells, which are known to contain L858R. The plasmid DNA was subcloned into a cloning Topo® vector (Invitrogen, Carlsbad, CA, USA). The experiments were carried out at a copy number of 10^7.

Results

Patients and pleural effusion specimens

Twenty-four patients with NSCLC were enrolled in the present study (Table 1). There were 11 women (45.8%) and 10 never-smokers (41.7%). The histological diagnosis was adenocarcinoma in 23 patients and unclassified NSCLC in the other patient. NSCLC was diagnosed cytologically in the pleural effusion samples in 22 of the patients. There were no malignant cells in the pleural effusion fluid of the other two patients. The age range was 39–82 years (median 62 years). Seven patients were treated with gefitinib (250 mg/day) and their response was evaluated. The volume of the pleural effusion fluid collected from the patients ranged from 30 to 280 mL. DNA from cell pellets was extracted for all 24 samples at concentrations ranging from 3.2 to 335.5 ng/μL.

Table 1. Patient characteristics and epidermal growth factor receptor mutation status

No.	Age (years)	Sex	Smoking history	Histology	Response to gefitinib	EGFR mutation	
						Direct sequencing	Scorpion ARMS
1	62	F	Never	ADC	PR	Wild type	E746_A750del
2	40	F	Never	ADC	SD	Wild type	Wild type
3	39	F	Never	ADC	PD	Wild type	Wild type
4	69	M	Former	ADC	–	Wild type	Wild type
5	72	F	Never	ADC	–	Wild type	Wild type
6	66	F	Never	ADC	–	Wild type	Wild type
7	56	M	Current	ADC	–	Wild type	Wild type
8	61	M	Former	ADC	–	Wild type	Wild type
9	65	M	Former	ADC	PD	Wild type	Wild type
10	80	F	Never	ADC	–	Wild type	E746_A750del
11	82	M	Current	NSCLC	–	Wild type	Wild type
12	57	F	Former	ADC	–	Wild type	Wild type
13	55	M	Former	ADC	–	Wild type	Wild type
14	67	M	Former	ADC	–	Wild type	Wild type
15	61	M	Never	ADC	PR	Wild type	E746_A750del
16	65	M	Former	ADC	PR	E746_A750del†	E746_A750del
17	65	F	Former	ADC	–	Wild type	L858R
18	48	F	Never	ADC	–	Wild type	L858R
19	61	M	Current	ADC	–	Wild type	Wild type
20	60	M	Current	ADC	PR	E746_A750del‡	E746_A750del
21	63	F	Never	ADC	–	E746_A750del‡	E746_A750del
22	54	M	Former	ADC	–	Wild type	Wild type
23	49	M	Current	ADC	–	Wild type	Wild type
24	66	F	Never	ADC	–	Wild type	Wild type

Type of mutation: †2236–2250del; ‡2235–2249del (NM_005228). –, Patient did not receive gefitinib; ADC, adenocarcinoma; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; SD, stable disease.

Sensitivity of direct sequencing and EGFR Scorpion for detection of E746_A740del

Preliminary experiments were carried out to evaluate the sensitivities of direct sequencing and the EGFR Scorpion Kit. When direct sequencing was used to detect E746_A750del in the standard E746_A750del DNA samples (10–10 000 pg), the mutation was detected at amounts as low as 10 pg. When diluted standard E746_A750del DNA was mixed with standard wild-type DNA at ratios from 1:1 to 1:1000, E746_A750del was detected by direct sequencing at ratios as low as 1:10.

When E746_A750del DNA was detected with Scorpion ARMS, all curves for standard E746_A750del DNA (10–10 000 pg) and the primer set for detection of E746_A750del increased for up to 45 cycles (Fig. 1A). When wild-type standard DNA and distilled water were used as negative controls, the curves did not increase, and remained flat at 50 cycles (Fig. 1A). When diluted standard E746_A750del DNA was mixed with wild-type DNA in ratios from 1:1 to 1:1000, all curves that indicated the presence of E746_A750del increased for up to 45 cycles (Fig. 1B). Standard curves in the range of measured amounts in this study were linear with R^2 values of 0.997 and 0.987. Both slopes of the curves were almost parallel (Fig. 1C). The Ct of diluted standard E746_A750del DNA mixed with wild-type DNA was almost the same as for standard E746_A750del DNA. Although the peak fluorescence levels of diluted standard E746_A750del DNA mixed with wild-type DNA were lower than without the wild-type DNA standard, the presence of E746_A750del was clearly detected at the ratio of 1:1000. Curves of DNA containing E746_A750del at amounts up to 10 pg were unaffected by interfusion of wild-type DNA.

The signals of plasmid DNA derived from the PC-9 cells and 11-8 cells were detected at approximately the same Ct values (E746_A750del, 28.6; L858R, 29.2) and, as expected, when plasmid DNA derived from A431 was used, the curve did not increase and remained flat after 50 cycles (Fig. 1D,E).

Detection of *EGFR* mutations by direct sequencing

EGFR mutations in three of the 24 patients (12.5%) were detected by direct sequencing (Table 1). All three were heterozygous, and E746_A750del was detected in all three of them. Figure 2 shows the wave figures of the nucleotide sequence obtained by direct sequencing of part of exon 19 in two patients (patient no. 10, Fig. 2A; patient no. 21, Fig. 2C). The data for patient no. 10 was judged to represent wild-type *EGFR* (Fig. 2A). That of patient no. 21 showed a mixture of wild-type and 2235–2249del sequences (Fig. 2C).

Mutation analysis using the Scorpion ARMS method

EGFR mutation status in all samples was analyzed using the EGFR Scorpion Kit. As wild-type curves were detected in all patients, we concluded that no sample was too small to be detect by the Scorpion ARMS method and that it would be possible to determine the *EGFR* mutation status based on the results. Curves for an *EGFR* mutation were detected in eight of the 24 patients (33.3%; Table 1). In six of these eight patients, curves indicating the presence of a deletion mutation

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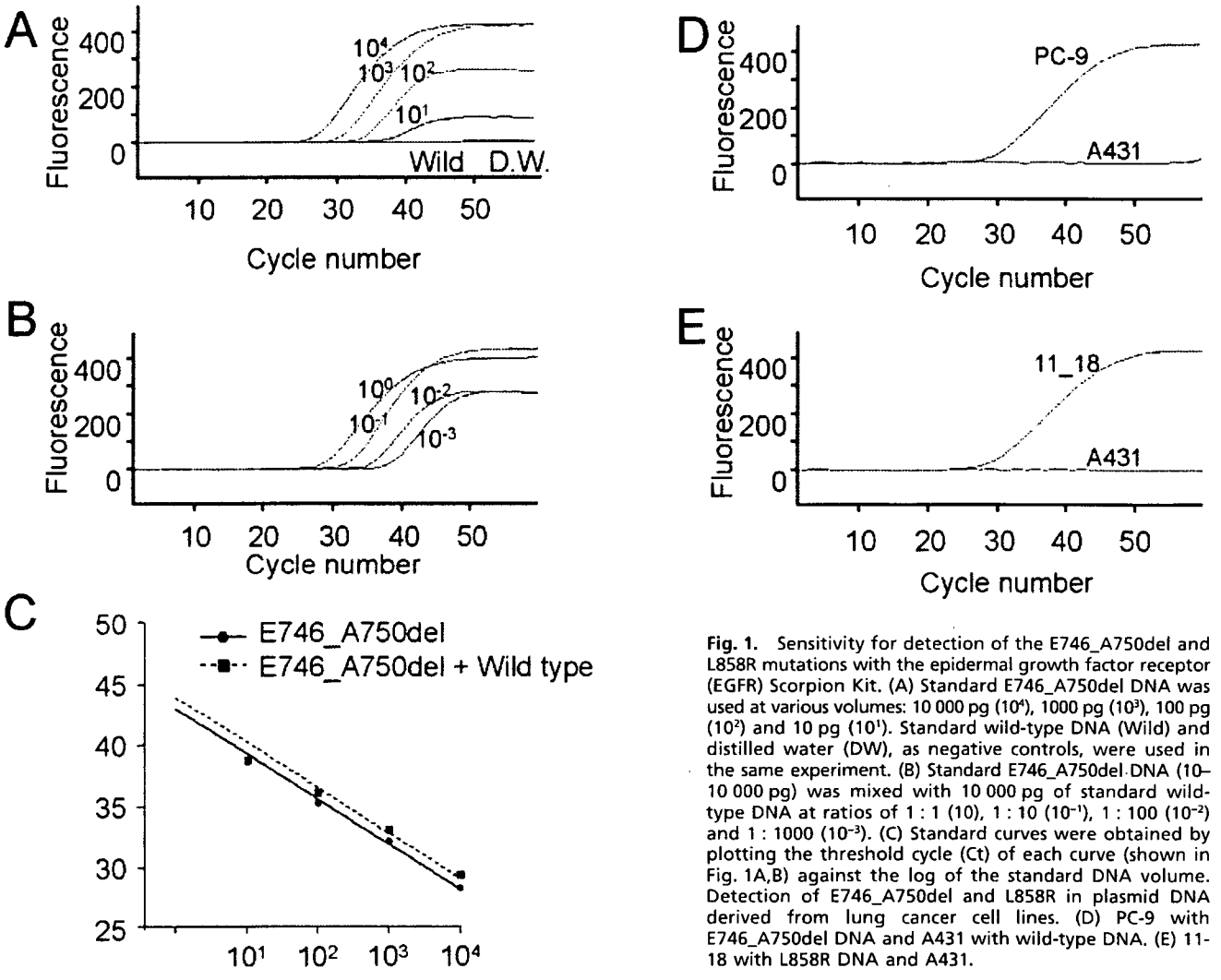

Fig. 1. Sensitivity for detection of the E746_A750del and L858R mutations with the epidermal growth factor receptor (EGFR) Scorpion Kit. (A) Standard E746_A750del DNA was used at various volumes: 10 000 pg (10^4), 1000 pg (10^3), 100 pg (10^2) and 10 pg (10^1). Standard wild-type DNA (Wild) and distilled water (DW), as negative controls, were used in the same experiment. (B) Standard E746_A750del DNA (10–10 000 pg) was mixed with 10 000 pg of standard wild-type DNA at ratios of 1 : 1 (10), 1 : 10 (10^{-1}), 1 : 100 (10^{-2}) and 1 : 1000 (10^{-3}). (C) Standard curves were obtained by plotting the threshold cycle (Ct) of each curve (shown in Fig. 1A,B) against the log of the standard DNA volume. Detection of E746_A750del and L858R in plasmid DNA derived from lung cancer cell lines. (D) PC-9 with E746_A750del DNA and A431 with wild-type DNA. (E) 11-18 with L858R DNA and A431.

in exon 19 were detected (Fig. 2B,D), and curves for the other two patients indicated the presence of L858R in exon 21.

Comparison of detection of the two major mutations by the two methods

In the present study *EGFR* mutations were detected in eight patients. In three of them (nos 16, 20 and 21) the *EGFR* mutations were detected by both methods, whereas in the other five (nos 1, 10, 15, 17 and 18) they were detected by the Scorpion ARMS method alone. No patients were found to have *EGFR* mutations by direct sequencing alone. *EGFR* mutations were not detected using either direct sequencing or the Scorpion ARMS method in two samples that were not diagnosed cytologically as NSCLC.

EGFR mutation status and clinical manifestations

EGFR mutations were detected more frequently in the samples from women (5/11, 45.5% of women; 3/13, 23.1% of men) and non-smokers (5/10, 50.0% of non-smokers; 3/14, 21.4% of smokers) (Table 2). Four of the seven patients who received gefitinib therapy had a partial response, one had stable disease, and the other three patients had progressive disease. All four patients with a partial response had *EGFR* mutations (Table 3). Evaluation of mutation status by the direct sequencing method revealed mutations in two of the four patients with partial response, whereas Scorpion ARMS revealed mutations in all four patients with partial response. Mutation status determined by Scorpion ARMS was superior to mutation status determined

Table 2. Frequency of *epidermal growth factor receptor (EGFR)* mutations in pleural effusion from patients with non-small cell lung cancer according to sex and smoking history

Variable	Direct sequencing		Scorpion ARMS	
	+	−	+	−
Sex and *EGFR* mutant state				
Female	1	10	5	6
Male	2	11	3	10
Smoking history and *EGFR* mutant state				
Non-smoker	1	9	5	5
Smoker	2	13	3	11

+, Mutation positive; −, no mutation; ARMS, Amplified Refractory Mutation System.

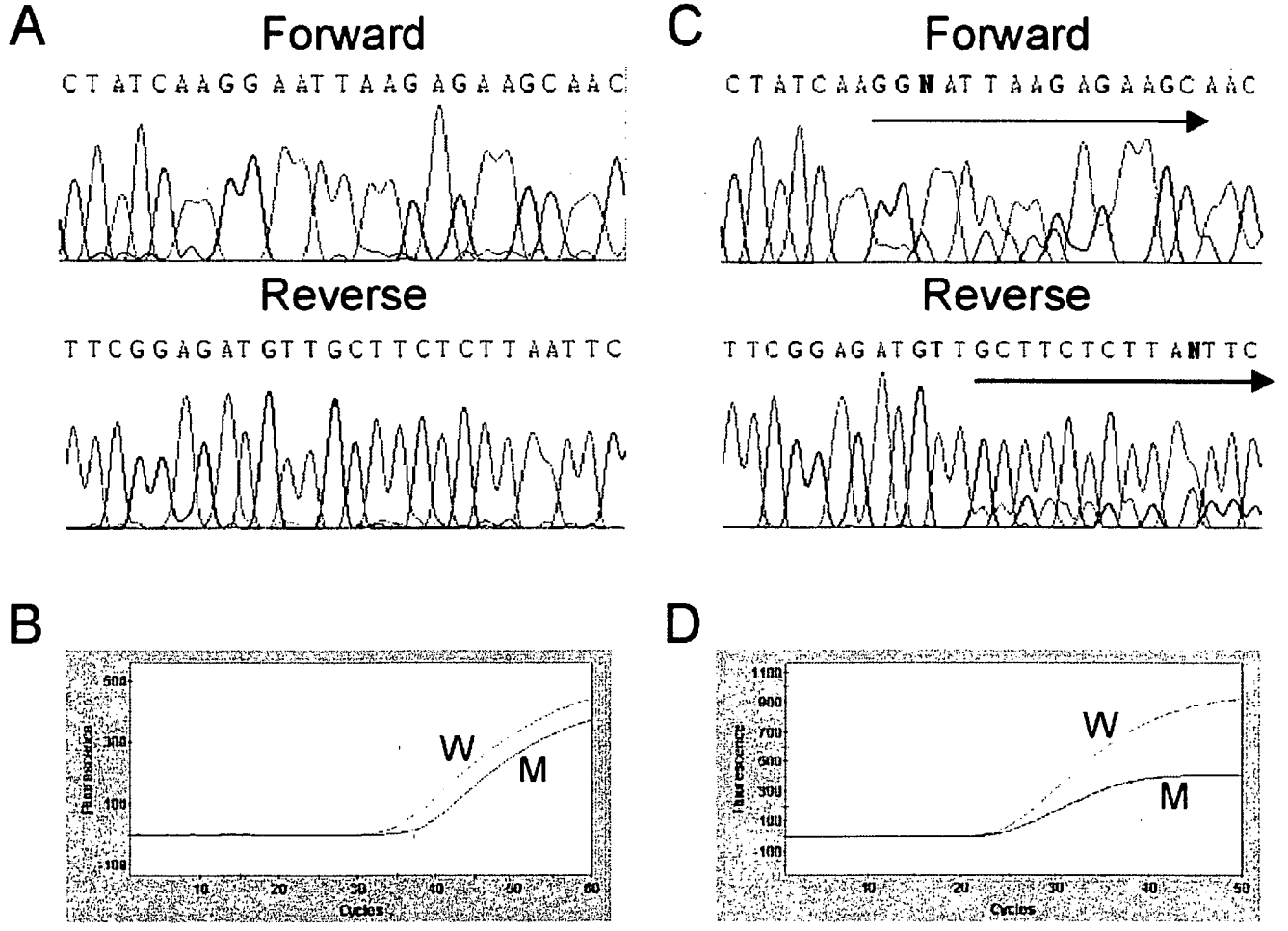

Fig. 2. Results of direct sequencing and the Scorpion Amplified Refractory Mutation System (ARMS) method in patient no. 10 (A,B) and patient no. 21 (C,D). (A) The wave figure represents wild-type *epidermal growth factor receptor (EGFR)*. (B) Two ascending curves, indicating that wild type and deletion mutation in exon 19 were detected. (C) The two waves start to overlap at the starting points of the arrows. These features mean that the nucleotide sequence of the *EGFR* gene in this patient has a heterozygous deletion. The deletion removed amino acids 746–750 (E746_A750del). (D) Two ascending curves, indicating that wild type and deletion mutation in exon 19 were detected.

Table 3. Frequency of *epidermal growth factor receptor (EGFR)* mutations in pleural effusion from patients with non-small cell lung cancer according to response to gefitinib

Variable	Direct sequencing		Scorpion ARMS	
	+	−	+	−
Partial response	2	2	4	0
Stable/progressive disease	0	3	0	3

The response to gefitinib was evaluated in all seven patients treated with gefitinib. +, Mutation positive; −, no mutation.

by direct sequencing for predicting responsiveness to gefitinib. No EGFR mutations were detected in patients with stable disease or progressive disease.

Discussion

The present study yielded two major findings. The first is that *EGFR* mutations, especially E746_A750 del and L858R, were detected in DNA from pleural effusion fluids, and the second is that the Scorpion ARMS method may be more sensitive for detecting *EGFR* mutations than the direct sequencing method. Patients with *EGFR* mutations may be misdiagnosed as not having any mutations if direct sequencing alone is used. Three patients were concluded to have mutations using both methods, but the other four patients were concluded to have mutations by the Scorpion ARMS method alone. As all four of these patients had partial responses to gefitinib, the results strongly suggest a correlation between mutation status and clinical responsiveness to gefitinib, although further clinical study is needed to make a definite conclusion. *EGFR* mutation status determined by the Scorpion ARMS method reflected responsiveness to gefitinib more accurately than direct sequencing.

Direct sequencing is currently the routine method used to detect *EGFR* mutations in tumor samples, and no standard method of detection of *EGFR* mutations in tumor specimens except surgical tissues has been established. The results of our small study lead us to conclude that the EGFR Scorpion Kit is superior to direct sequencing for detection of *EGFR*

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mutations, especially the two major mutations (deletion mutations in exon 19 and L858R), as predictive markers. As our preliminary experiment showed that the sensitivity of Scorpion ARMS for detection of *EGFR* mutations is superior to the sensitivity of direct sequencing when a mixture of wild-type and mutant DNA is used, we infer from these results that the differences in sensitivity for detection in the four patients with the mutations were attributable to the density of tumor cells in the pleural effusion fluid.

To our knowledge detection of *EGFR* mutations in pleural effusion fluid has been described in one case report where the patient responded to gefitinib.[15] Although our study did not confirm a correlation between mutation status and clinical responsiveness to EGFR tyrosine kinase inhibitors such as gefitinib, their results and our own in patients who received gefitinib therapy encourage us to conclude that *EGFR* mutation status determined in pleural effusion fluid may be useful for predicting responsiveness to EGFR tyrosine kinase inhibitors. The authors of the case report did not mention the possibility that normal cells may have prevented detection of *EGFR* mutations in tumor cells and that a patient with an *EGFR* mutation may be concluded not to have a mutation (false negative) as a result.

Some investigators have tried to increase the sensitivity of *EGFR* mutant detection. One attempt involved detection of *EGFR* mutations using a LightCycler PCR assay.[16] SSCP assay is more sensitive than direct sequencing and is a more rapid method.[17] Recently, two rapid and sensitive methods have been demonstrated: the peptide nucleic acid-locked nucleic acid PCR clamp method,[18] and the mutant-enriched PCR assay.[19] In these previous studies, *EGFR* mutations were detected in the presence of 1000-fold and 2000-fold wild-type *EGFR*, respectively. Although the minimum detectable mutation volumes were not evaluated, the sensitivity of these methods seems to be comparable with that of the Scorpion ARMS method, and the sensitivity of these assays seems to be sufficient for clinical use. The latter study used various clinical samples, including 20 samples of pleural fluid. We have shown a relationship between *EGFR* mutation status in pleural fluids and the gefitinib response in a portion of the enrolled patients. The relationship in the remaining patients is currently being evaluated, and confirmation is expected in the very near future. As the Scorpion ARMS method is simple and very fast, it may be suitable for mutation screening. However, one limitation of the EGFR Scorpion Kit is that it is only able to detect mutations targeted by the Scorpion primers. It is known that deletion mutations in exon 19 have many variations in deleted nucleotides and addition of point mutations. The Scorpion ARMS method could detect mutations targeted by primers designed in advance and is capable of detecting the specific mutation E746_A750del in exon 19. E747_P753del insS and L747_T751del are minor variations of deletion mutations in exon 19 and could not be detected using this method in another study (data not shown). All *EGFR* mutations are not at these two sites; some are clustered around the ATP-binding site in exons 18, 19 and 21.[3–8] Although approximately 90% of NSCLC-associated *EGFR* mutations consist of the two major EGFR mutations,[5,8] other mutations may be misdiagnosed as negative mutation results using the Scorpion ARMS method. Moreover, a secondary mutation, a substitution of methionine for threonine at position 790 (T790M), leads to gefitinib resistance in NSCLC patients who have *EGFR* mutations and are responsive to gefitinib.[20,21] These mutation states may also be critical factors for gefitinib therapy. Scorpion primers need to be designed to detect these mutations, and further study using these primers is required.

Our two initial aims, which were to detect two major EGFR mutations in pleural effusion fluid and to increase the sensitivity of detection of *EGFR* mutations in the mixtures of DNA from normal cells and tumor cells, were achieved in this study. As the next step, a prospective study of a large number of NSCLC patients with pleural effusion is likely to reveal a correlation between *EGFR* mutation state in pleural effusion fluids and clinical responsiveness to EGFR tyrosine kinase inhibitors, such as gefitinib.

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EGFR Mutation of Tumor and Serum in Gefitinib-Treated Patients with Chemotherapy-Naive Non–small Cell Lung Cancer

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Background: The authors evaluate the efficacy and safety of gefitinib monotherapy in chemotherapy-naive patients with advanced non–small-cell lung cancer (NSCLC). A secondary endpoint is to evaluate the relationship between clinical manifestations and epidermal growth factor receptor (*EGFR*) mutation status.

Methods: Japanese chemotherapy-naive NSCLC patients were enrolled. They had measurable lesions, Eastern Cooperative Oncology Group performance status of 0 to 2, and adequate organ and bone marrow function. Patients received 250 mg of oral gefitinib daily. *EGFR* mutations in exon 18, 19, and 21 of DNA extracted from tumor and serum were analyzed by genomic polymerase chain reaction and direct sequence.

Results: All 30 patients were eligible for the assessment of efficacy and safety. An objective response and stable disease were observed in 10 patients (33.3%) and nine patients (30.0%), respectively. The median time to progression was 3.3 months and the median overall survival was 10.6 months. The 1-year survival rate was 43.3%. Grade 3 toxicities were observed in seven patients. *EGFR* mutation was observed in four of 13 (30.8%) tumors, and two of them achieved partial response. In serum samples, three of 10 patients with *EGFR* mutations in the serum before treatment had a response to gefitinib. *EGFR* mutation was observed in 10 of 27 and significantly more frequently observed in the posttreatment samples from patients with a partial response or stable disease than in those from patients with progressive disease ($p = 0.006$).

Conclusions: Gefitinib monotherapy in chemotherapy-naive NSCLC patients was active, with acceptable toxicities. These results warrant further evaluation of gefitinib monotherapy as a first-line therapy. The *EGFR* mutation in serum DNA may be a biomarker for monitoring the response to gefitinib during treatment.

Key Words: Non–small-cell lung cancer, Gefitinib, Epidermal growth factor receptor, Mutation, Serum DNA.

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Non–small-cell lung cancer (NSCLC) is the leading cause of cancer death in Japan and throughout the world.[1] Unfortunately, the majority of patients with NSCLC present with locally advanced or metastatic disease at the time of diagnosis. Although chemotherapy has produced modest survival benefits in advanced NSCLC patients, the outcome of chemotherapy for NSCLC remains unsatisfactory.

Protein tyrosine kinases play important roles in the pathogenesis of malignant tumors.[2] Among them, epidermal growth factor receptor (EGFR) tyrosine kinase has been implicated in the initiation and progression of NSCLC.[3–5] The overexpression of EGFR is frequent in NSCLC.[6] Monoclonal antibodies and low-molecular-weight compounds that inhibit the EGFR signaling pathway have been developed and shown to have antitumor effects. Gefitinib (Iressa, AstraZenca, London, England) is an orally active EGFR type tyrosine kinase inhibitor. In four phase I studies, tumor shrinkage or stabilization after gefitinib monotherapy was observed in some patients with NSCLC. In two phase II trials, Iressa Dose Evaluation in Advanced Lung cancer (IDEAL) 1 and 2, gefitinib monotherapy was shown to have a substantial effect in NSCLC patients treated previously with chemotherapy.[7,8] In these trials, patients of Asian origin and who had never been smokers had a statistically significant improvement in overall survival. In spite of encouraging results in the IDEAL trials, two large-scale, phase III, randomized trials, Iressa NSCLC Trial Assessing Combination Treatment, failed to show any survival benefit for the use of gefitinib.[9,10] Patients in a large-scale phase III trial comparing gefitinib

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